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OCCURRENCE OF CHOLINE ACETYLTRANSFERASE AND ACETYLCHOLINE AND OTHER QUATERNARY AMMONIUM COMPOUNDS IN MAMMALIAN SPERMATOZOA

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Abstract—These investigations were undertaken to show the presence of acetylcholine (ACh) and other quaternary ammonium compounds and choline acetyltransferase (ChA) activity in mammalian spermatozoa. The spermatozoa from the fresh ejaculates of the bull and man were washed with calcium-free Krebs–Ringer phosphate solution and extracted with acetonitrile. The quarternary ammonium compounds from the extracts were subjected to pyrolysis gas chromatography. In the gas chromatogram of the quaternary ammonium compounds of bull spermatozoa, two peaks were found which represented 2-dimethylaminoethyl acetate and 2-dimethylaminoethyl propionate. In the gas chromatogram of the quaternary ammonium compounds of the human spermatozoa, a peak for 2-dimethylaminoethyl acetate was found. These observations suggest that ACh and propionylcholine (PCh) occur in bull spermatozoa and that ACh occurs in human spermatozoa. Bull spermatozoa contained 4.27 $\pm$ 1.41 pmoles (mean $\pm$ S.E.M.) ACh/10^6 cells and 1.47 $\pm$ 0.48 pmoles PCh/10^6 cells, and human spermatozoa contained 28.57 $\pm$ 3.38 pmoles ACh/10^6 cells. ChA activity was determined in the washed spermatozoa by a radiometric assay. Incubation of the homogenates of the sperm cells with [^{14}C]acetylcoenzyme A and choline resulted in the synthesis of [^{14}C]ACh, which was separated, using a column of an anion exchange resin, and assayed. ChA activity in bull spermatozoa was 200.19 $\pm$ 32.08 pmoles ACh synthesized/10^6 cells/10 min, and ChA activity in human spermatozoa was 130.90 $\pm$ 14.19 pmoles ACh synthesized/10^6 cells/10 min. The specific activity of ChA in the tails of bull spermatozoa was about five times higher than in the heads or midpieces.

The existence of a cholinergic system consisting of the acetylcholine (ACh) cycle (synthesis, stimulation at a receptor, and hydrolysis by cholinesterase) at various sites of the nervous system has been well established. Recent investigations indicate that the ACh cycle plays a significant role in non-nervous tissues, one of which is spermatozoa [1–3].

Szent-Gyorgyi [4] developed the glycerine fiber muscle model as a standard experimental system in the field of muscle pharmacology. Subsequently, this model was extended to the study of non-muscular systems by a number of investigators [5, 6]. Nelson [7] reviewed the evidence that contractile proteins were responsible for movement in a variety of cells and organelles, including spermatozoa. They were described as actin-like, myosin-like and actinomysin-like proteins which displayed many of the biochemical and physiological parameters of contractile proteins of the muscle. The motility of spermatozoa was compared with the automaticity of the smooth muscle, and it was suggested that an ACh cycle might be responsible for the contraction and relaxation cycles of spermatozoa.

Although detailed studies are not available, the occurrence of ACh-like substances in the cytoplasm of the rabbit, bull, ram and boar spermatozoa was demonstrated by Saiko [8, 9], using bioassay techniques. Adding ACh increased the motility of the spermatozoa from the *Arbacia punctulata* minimally but significantly [10]. In the presence of dimethylsulfoxide (DMSO), which increases the permeability of membranes to quaternary ammonium compounds, ACh increased the motility of spermatozoa in low concentrations (10^{-5} to 5×10^{-4} M) and inhibited the motility in high concentrations (10^{-3} to 10^{-2} M).

The presence of a cholinesterase-like enzyme has been demonstrated in spermatozoa of several mammalian and marine invertebrate species [11–14]. The enzyme of the pig and bull was identified as acetylcholinesterase (AChE) by using specific substrates and inhibitors. The enzyme selectively hydrolyzed ACh but was less effective at hydrolyzing butyrylcholine and benzoylcholine. The enzymatic hydrolysis of ACh showed an optimum substrate concentration of about 1.4×10^{-2} M. Physostigmine was a competitive inhibitor of bull sperm AChE. Ethylmercuric thiosalicylate (Merzonine), which inhibited horse serum ChE, did not inhibit pig sperm AChE.

All parts of spermatozoa (head, midpiece and tail) contain significant amounts of AChE. In the bull spermatozoa, the tails contain higher concentrations of AChE than the heads or midpieces [13]. AChE in ram spermatozoa was largely confined to the tail fragments [15], while it occurred mostly in the heads of trout and perch spermatozoa [12].

Nelson [16] postulated that AChE may regulate sperm motility by controlling intracellular levels of ACh. Physostigmine increased the motility of *A. punctulata* spermatozoa at low concentrations (10^{-6} M) and inhibited their motility at high concentrations

(10^{-3} M). Neostigmine and tetraethylammonium, which do not pass through membrane barriers, produced minimal changes in motility.

Hemicholinium, a pharmacologic agent which blocks the uptake of choline into acetylcholine-synthesizing cells, inhibited sperm motility. In the presence of DMSO, the effectiveness of hemicholinium increased 2-fold. While junctional-like structures have not been identified in spermatozoa by ultrastructure techniques, evidence suggesting the presence of a junctional structure has been presented by Nelson [10]. Curare, which inhibits ACh binding to a postsynaptic receptor, produced effects ranging from about 20 per cent acceleration of motility at 5×10^{-4} M to 20 per cent inhibition at 5×10^{-3} M. Strychnine, which blocks postsynaptic inhibition of Renshaw cells, exerted similar effects ranging from 40 per cent stimulation at 10^{-5} M to about 40 per cent inhibition at 5×10^{-3} M.

Trimethyl phosphate administered to male rabbits and rats caused sterility [17]. The spermatozoa from the treated animals exhibited abnormal motility. Choline acetyltransferase (ChA) activity in the spermatozoa from animals treated with trimethyl phosphate was demonstrated to be lower than controls.

Among the three components of the ACh cycle, synthesis of ACh and its occurrence in spermatozoa have been studied the least. These investigations were undertaken to demonstrate the presence of ACh and other quaternary ammonium compounds in human and bull spermatozoa. The distribution of ChA-like activity in subcellular fractions of these spermatozoa was also studied.

METHODS

Bull ejaculates were collected by an artificial vagina and cooled immediately to 4°. All ejaculates were pooled and used within 2 hr of collection. Ejaculates were washed three times with cold (4°) calcium-free Krebs–Ringer phosphate solution and counted with a Spencer Bright-line hemacytometer in 0.1% formalin.

Human ejaculates were collected from normal healthy volunteers. Ejaculates were allowed to liquefy at room temperature (25°) before being pooled and washed three times with cold (4°) Norman–Johnson's solution without glucose [18].

Extraction of quaternary ammonium compounds. Sperm pellets, containing in excess of 5×10^7 and 10^8 cells/cm^3 (human and bull, respectively), were resuspended with 1 ml diluent. An internal standard was added to each suspension (propionylcholine and valerylcholine, respectively) for quantitation.

The method for extraction of quaternary ammonium compounds was modified from the procedure described by Schmidt and Speth [19]. Sperm suspensions were homogenized with a Polytron PT 10 homogenizer at maximum speed for 20 sec and 9 ml acetonitrile. Extracts were centrifuged and the sediment was discarded. To the ACh extract 10 ml of an ether–toluene extract system (1:1) was added, the tubes were shaken for 2 min, and the solvent was aspirated off. This step was repeated before blowing off the remaining solvent with N_2, which left an aqueous sperm extract of quaternary ammonium compounds. The aqueous sperm extract was frozen (−70°) and lyophilized overnight. In order to avoid the interference by endogenous choline, it was esterified by adding 0.05 ml acetonitrile (containing 0.05 ml butyrylchloride) to the lyophilized extract and then heating the mixture at 60° for 60 min. Choline esters, as well as other quaternary ammonium compounds, were precipitated with 0.2 ml potassium iodide–iodine solution (10 g KI, 9 g I_2 in 50 ml H_2O) in the presence of tetramethylammonium (1 μg/μl of acetonitrile). The precipitate was isolated by centrifugation and analyzed by pyrolysis gas chromatography.

Pyrolysis gas chromatography. The quaternary ammonium compounds were analyzed according to the procedure described by Schmidt and Speth [19]. During pryolysis, the quaternary ammonium compounds were converted into their tertiary analogs, whose peaks appeared in the gas chromatogram.

The assays were run on a Fisher Victoreen No. 4400 gas chromatograph equipped with a Nuclear Chicago pyrolyzer. The aluminum column was 8 ft × $\frac{1}{4}$ in. in size and packed with 20% carbowax 6000 on 60/80 mesh Chromosorb W (HMDS). Gas flows were: N_2, 65 ml/min; O_2, 2000 ml/min; H_2, 30 ml/min. Temperatures were: column, 140°; pyrolyzer, 160°; injection port, 180°; and flame ionization detector, 210°.

Choline acetyltransferase assay. The radiometric assay described by Sastry and Henderson [1] was modified and applied to the determination of the ChA activity in the sperm. The sperm enzyme preparation was made by adding a known quantity of washed spermatozoa to an equal volume of cold 0.02 M sodium bicarbonate buffer (pH 7.4) (containing EDTA, 5.0 mM; NaCl, 0.3 M; and NaCN, 20 mM) and homogenized with a Polytron PT 10 homogenizer for 15 sec at maximum speed. ChA-like activity in the sperm homogenate was determined by the conversion of [^{14}C]ACoA and choline to [^{14}C]ACh. The reaction mixture (0.3 ml) was composed of (a) 0.1 ml of substrate mixture, which brought the final concentration to 0.3 M NaCl, 2×10^{-4} M physostigmine sulfate, 0.02 M $MgSO_4$, 10^{-4} M choline and 10^{-5} M labeled [^{14}C]ACoA and unlabeled ACoA; (b) 0.1 ml of 0.05 M K_2PO_4 buffer (pH 7.4); and (c) 0.1 ml of the sperm homogenate. Reactions were run at 37° for 10 min and stopped by chilling the reaction tube in crushed ice. The [^{14}C]ACh was separated from the ^{14}C-substrate by applying 0.1 ml of the incubation mixture to a column of anion exchange resin (BioRad, AG1-X8; 1.3 g dry weight), followed by 2.0 ml distilled water, added in successive 4 × 0.5 ml portions. The eluate was collected in a scintillation vial to which 15 ml 'fluor' (*p*-dioxane containing 0.5% of 2,5-diphenyloxazole and 10% naphthalene) was added. The vial was counted in a Packard Tricarb scintillation counter for 10 min and the ^{14}C yield was correlated to ACh production. Values were expressed as pmoles ACh produced/10^6 cells/10 min of incubation time.

RESULTS

Gas chromatographic spectra of bull and human spermatozoa. The spectrum of bull spermatozoa is represented in Fig. 1. Spectrum A shows three peaks corre-

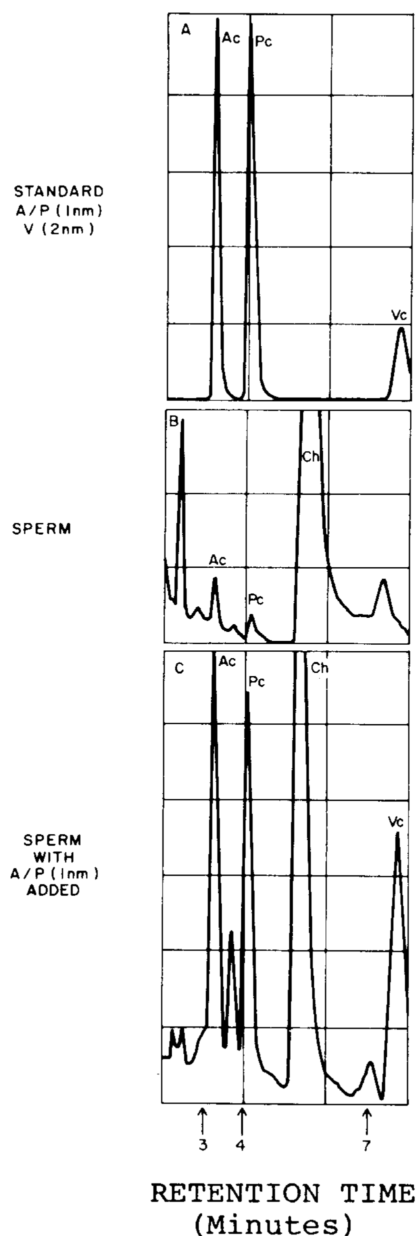

Fig. 1. (A) Pyrolysis gas chromatogram of standard ACh and PCh (1 nmole each) and VCh (2 nmoles). The peaks of the tertiary amines formed from ACh, PCh and VCh are labeled Ac, Pc and Vc respectively. (B) Gas chromatograms resulting from the pyrolysis of the quaternary ammonium compounds from the bull sperm extract. Peaks that were identified are the tertiary analogs of ACh (Ac), PCh (Pc) and choline (Ch), which formed during pyrolysis. (C) Pyrolysis gas chromatogram from the same sperm samples as in (B) but with added ACh and PCh (1 nmole each) and VCh (2 nmoles). Sensitivity: 64×10^{-11}.

sponding to dimethylaminoethyl acetate (Ac), dimethylaminoethyl propionate (Pc) and dimethylaminoethyl valerate (Vc), which are products from pyrolysis of a standard mixture of ACh, propionlycholine (PCh) and valerylcholine (VCh). Spectrum B represents a gas chromatogram after pyrolysis of the extract of bull spermatozoa. Two peaks (marked Ac and Pc) have the same retention times as those of 2-dimethylaminoethyl acetate and 2-diethylaminoethyl propionate seen in spectrum A. A peak corresponding to VCh did not appear in the bull sperm extracts. Choline occurs in bull sperm and is represented as dimethylaminoethyl butyrate (Ch). When ACh (1.0 nmole) and PCh (1.0 nmole) were added to a duplicate sample of bull sperm extract, the peak heights of Ac and Pc increased (spectrum C). These two compounds, therefore, occur in bull spermatozoa.

Extracts from human spermatozoa were analyzed in the same manner. Figure 2 represents the gas chromatogram after pyrolysis of the human sperm extract. Spectrum A in Fig. 2 represents control samples of ACh and PCh. Pyrolysis of human sperm extract (spectrum B) gave one peak with the same retention time as that of the control ACh (Ac). The addition of 1 nmole ACh (spectrum C) increased the height of the Ac peak. These observations indicate that human sperm contains ACh but not PCh. As in the

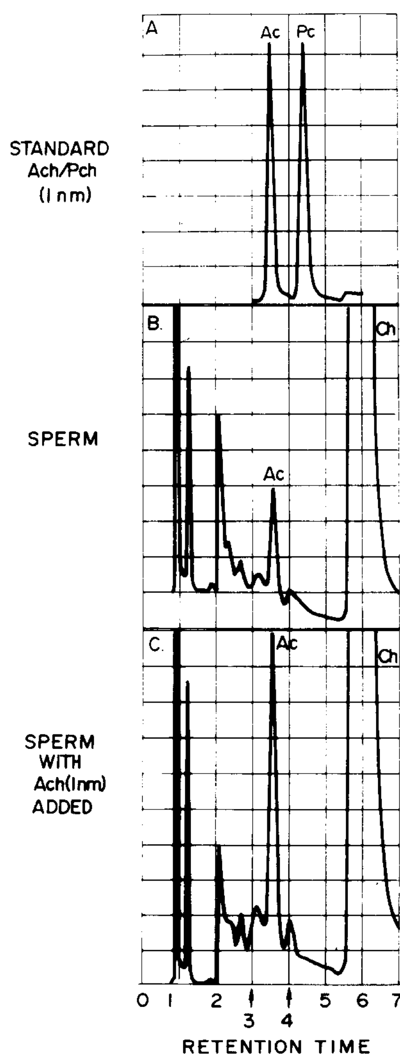

Fig. 2. (A) Pyrolysis gas chromatogram of standard ACh and PCh (1 nmole each). Peaks of 2-dimethylaminoethyl acetate and 2-dimethylaminoethyl propionate were labeled as Ac and Pc respectively. (B) Pyrolysis gas chromatogram of human sperm extract. (C) Gas chromatogram of pyrolysis of human sperm extract, duplicate sample to (B), with ACh (1 nmole) added. Sensitivity: 32×10^{-11}.

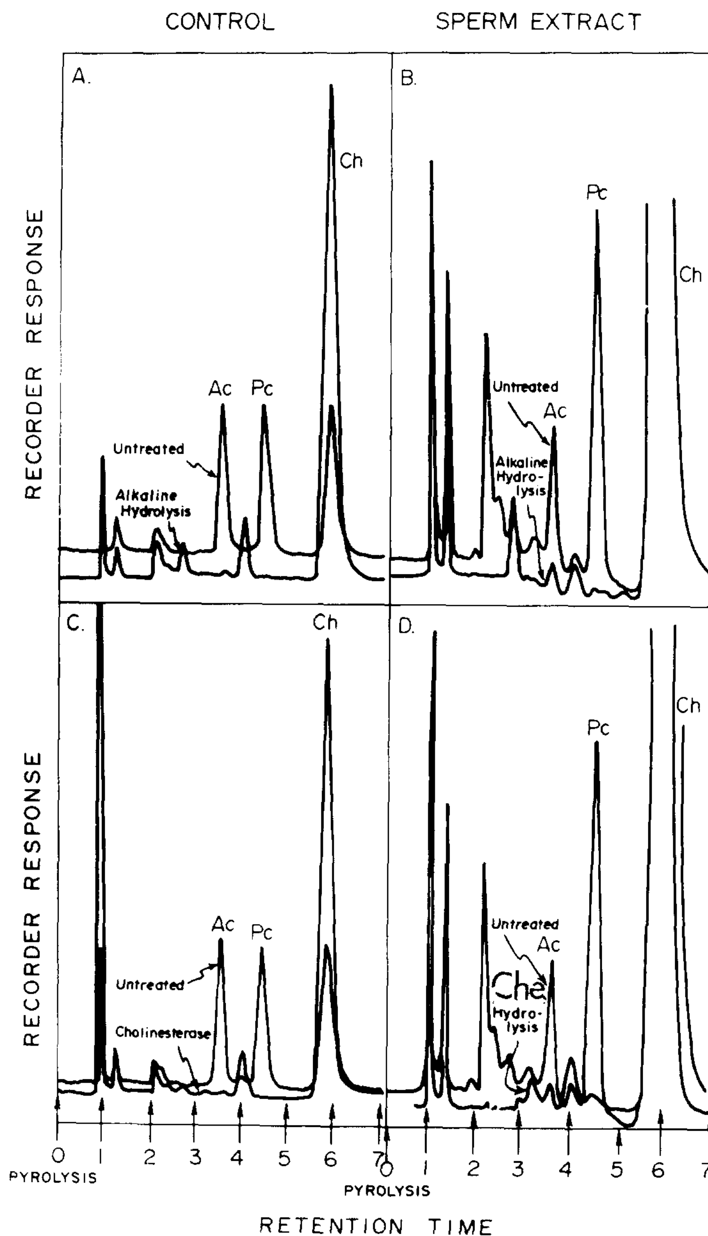

Fig. 3. (A) Pyrolysis gas chromatograms of ACh and PCh (5 nmoles each) with and without alkali treatment. (B) Pyrolysis gas chromatograms of human sperm extract with and without alkali treatment. (C) Pyrolysis gas chromatogram of the standard sample, ACh and PCh (5 nmoles each), with and without excess electric eel AChE treatment. (D) Pyrolysis gas chromatograms of human sperm extract with and without excess electric eel AChE. Sensitivity: A and C, 128×10^{-11}; B and D, 32×10^{-11}. In all four panels, the upper tracing indicates the pyrolysis gas chromatogram of the sample before chemical treatment, and the lower tracing indicates the pyrolysis chromatogram of the same sample after the alkaline or enzymatic treatment.

bull sperm, the human sperm contains abundant amounts of choline (Ch).

Influence of alkaline hydrolysis and AChE hydrolysis of the extracts of spermatozoa on their gas chromatograms. Hydrolysis by alkali treatment or by electric eel AChE treatment has been used by other investigators to identify the presence of ACh and other choline esters in extracts of biological samples [20–22]. Alkalinization of sperm extracts to pH 12 with 1 N NaOH, before the esterification step of choline, and subsequent incubation at 60° for 30 min, should remove the Ac, Pc and Vc peaks. Similarly, addition of eel AChE to the sperm extract and incubation of the mixture at 37° for 30 min should remove the Ac, Pc and Vc peaks, if the corresponding compounds are substrates for the enzyme.

After hydrolysis of human sperm extracts with alkali or electric eel AChE (Fig. 3), the height of the peak representing ACh (Ac) was significantly reduced (80 per cent) in the control samples, which contained 5 nmoles each of ACh and PCh (spectra A and C) as well as in the sperm extracts (spectra B and D).

Table 1. Acetylcholine (ACh) and propionylcholine (PCh) in mammalian spermatozoa

Species	ACh*	PCh*
Bull	4.27 ± 1.41	1.47 ± 0.48
Human	28.57 ± 3.38	

* Expressed as pmoles (mean ± S.E.M.)/10^6 cells.

In the spectra of the extracts of bull spermatozoa which were subjected to alkaline hydrolysis or AChE treatment, the two peaks corresponding to ACh and PCh (Ac and Pc) were absent. This suggests that endogenous ACh and PCh were hydrolyzed by alkali or AChE and, therefore, the corresponding peaks disappeared from the spectrum.

Content of ACh and PCh in spermatozoa. Choline esters were quantified in bull and human spermatozoa by preparing the cells as mentioned in the above methods. Before homogenizing the sperm in ACh, valerylcholine (2 nmoles) and propionylcholine (1 nmole) were added to the bull and human sperm, respectively, as internal standards. The ratio between internal standard and ACh or PCh yielded the quantities in nmoles. In all cases sufficient numbers of cells were used to allow at least 1 nmole to be analyzed on the gas chromatograph.

Table 1 gives the amount of ACh and PCh in bull and human spermatozoa. Human sperm contains higher amounts of ACh than the bull sperm. There is no evidence for the presence of PCh in human sperm. The ratio of ACh:PCh in bull sperm was 3:1.

Influence of storage on sperm ACh content. The occurrence of ACh in membrane stores in nervous tissue is well established. In order to analyze for membrane stores of ACh in the sperm, fresh, washed bull spermatozoa were divided into two fractions. One fraction was refrigerated at 4° for 2 days and the other fraction was frozen for 2 days at −12°. If ACh is separated from AChE by a membrane, then the ACh peak should remain intact in the pyrolysis gas chromatogram of the sperm extract. After freezing, the membranes will be disrupted, due to the formation of ice crystals. If the frozen sperm is thawed, ACh will be hydrolyzed by AChE. Therefore, the ACh peak should be absent in the gas chromatogram of the frozen sperm. In the pyrolysis gas chromatograms of extracts of the refrigerated or frozen sperm, peaks corresponding to ACh and PCh were absent. These experiments indicate that ACh does not exist in a stored form within the membranes of the sperm.

Choline acetyltransferase-like activity in bull and human sperm. Both species contain ChA-like activity (Fig. 4). Bull sperm has greater ChA-like activity than human sperm were 200.19 ± 32.07 and 130.90 ± 14.19 pmoles ACh formed/10^6 cells/10 min respectively.

Distribution of choline acetyltransferase activity in spermatozoa. The ChA-like activities in the three sperm fractions (head, midpiece and tail) were determined by subjecting a suspension of washed bull spermatozoa to 20 kc of ultrasonic vibration and separating the fractions by centrifugation, as described by Nelson [13]. A 90–95 per cent pure preparation was obtained with this method. In Fig. 5, the clear bars represent the specific activity of ChA (in pmoles ACh formed/mg of protein/10 min) and the bars with diagonal lines indicate AChE activity (in μg ACh breakdown/mg of protein/hr), as reported by Nelson [13] in the sperm activity. Specific activities of both ChA and AChE of the tail fraction were about five times higher than the corresponding values for the head or the midpiece.

DISCUSSION

The gas chromatograms presented have indicated the occurrence of ACh in both human and bull spermatozoa. Bull spermatozoa also contain PCh. The peaks representing the presence of ACh and PCh in sperm extracts were further identified by mass spectrometry as those of the demethylated compounds of ACh and PCh (unpublished results from our laboratory). There is a 7-fold difference between the quanti-

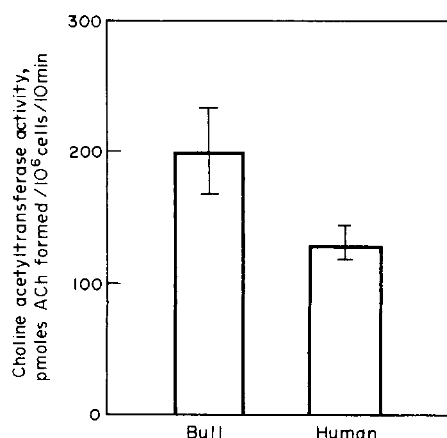

Fig. 4. Choline acetyltransferase (ChA) activities in bull and human sperm.

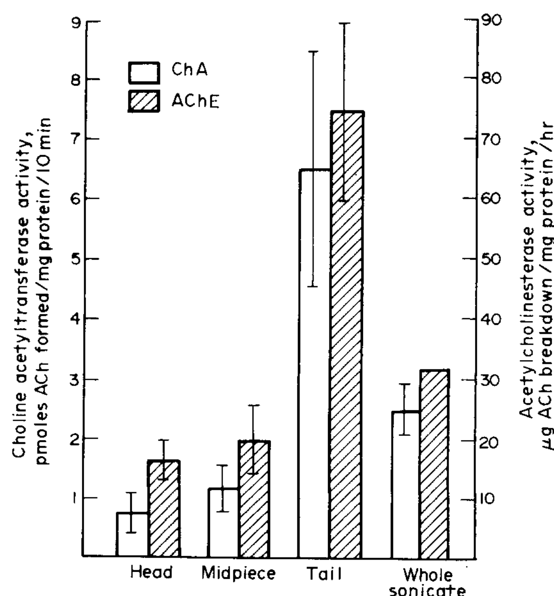

Fig. 5. Choline acetyltransferase (ChA) and acetylcholinesterase (AChE) distribution in bull sperm fractions. AChE values were quoted from Nelson [13].

ties of ACh in bull and human sperm. The experimental values for the bull sperm should be considered as lower than the normal values. A contributing factor might be the immediate availability of a homogeneous population of human donors unlike the population of bull donors. Although the bull ejaculates were immediately cooled to 4°, they were transported several miles to our laboratory before they were analyzed. A second contributing factor for this difference is species variation. Human spermatozoa do not contain PCh, while the bull spermatozoa contain significant quantities of PCh. There is no significant difference in the pH values of semen from both species [23]. However, the AChE activity of human spermatozoa was considerably lower than that of the bull. Our results indicate that ChA activity in bull spermatozoa is greater than the ChA activity of human spermatozoa. These observations indicate that the rates of synthesis and hydrolysis were higher in the bull spermatozoa than those in the human spermatozoa.

Our results support the theory that a ChA–ACh–AChE system plays a significant role in insuring and controlling the motility of the spermatozoa. The lack of membrane stores for ACh indicates that ACh synthesis, the stimulation of a receptor by ACh, and the hydrolysis of ACh by AChE are closely linked and may be localized within the same cell compartment. This is supported by the observation that the distribution of ChA and AChE in subcellular fractions of bull spermatozoa is similar. In view of the lack of stores for ACh in spermatozoa, it is not possible to determine its subcellular distribution.

Many factors are important for motility. Further characterization of an ACh cycle may give more insight into this process. Available evidence indicates that there is a cholinergic receptor of the nicotinic type in the spermatozoa. Therefore, investigations are being undertaken in our laboratory to isolate and characterize the ACh-binding proteins in spermatozoa with ^{125}I-α-bungarotoxin, which is known to bind to nicotinic receptors. A successful development of specific ChA inhibitors which would exhibit selective permeability into the testicular tissue may result in the development of a useful male contraceptive.

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